

METHOD FOR PREDICTING THE RESPONSE TO HER2-DIRECTED THERAPY

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BACKGROUND OF THE INVENTION

1. **Field of the Invention**

This invention relates to methods for predicting the response to HER2-directed therapy in an individual.

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2. **Background of the Invention**

Cellular growth and differentiation processes involve growth factors that exert their actions through specific receptors expressed in the surfaces of responsive cells. Ligands binding to surface receptors, such as those that carry an intrinsic tyrosine kinase activity, 15 trigger a cascade of events that eventually lead to cellular proliferation and differentiation (Carpenter *et al.*, 1979, Biochem., 48: 193-216; Sachs *et al.*, 1987, Cancer Res., 47: 1981-1986). Receptor tyrosine kinases can be classified into several groups on the basis of sequence similarity and distinct features. One of these groups includes the epidermal growth factor receptor family, which includes erbB-1 (also termed EGFR or HER-1) (Carpenter *et al.*, *supra*); erbB-2 (HER-2/neu) (Semba *et al.*, 1985, Proc. Natl. Acad. Sci., 82: 6497-6501; Coussens *et al.*, 1985, Science, 230: 1130-1139; Bargmann *et al.*, 1986, Cell, Vol. 45, 649-657); erbB-3 (HER-3) (Kraus *et al.*, 1989, Proc. Natl. Acad. Sci., 86: 9193-9197; Carraway *et al.*, 1994, J. Biol. Chem., 269: 14303-14306), and erbB-4 (HER-4) (Plowman *et al.*, 1993, Nature, 366: 473-475; Tzahar *et al.*, 1994, J. Biol. Chem., 269: 25226-25233).

25 Most tumors of epithelial origin express multiple erbB (HER) receptors and co-express one or more EGF-related ligands suggesting that autocrine-receptor activation plays a role in tumor cellular proliferation. Because these ligands activate different erbB/HER receptors, it is possible that multiple erbB receptor combinations might be active in a tumor, a characteristic that could influence its response to an erbB-targeted therapeutic. ErbB receptors form homodimers and heterodimers that can be stimulated by various ligands 30 leading to downstream signaling events, the extent and nature of which depend on the

combination of specific dimers and ligands. For example, HER2/neu appears to be the preferred heterodimerization partner with other members of the epidermal growth factor receptor family, but ultimately the dimers formed are determined by the ligand and the erbB receptors expressed in the cell. Not only may the ligand select the dimerization partners, but
5 it may also influence the time course of membrane translocation, activation, and internalization of the receptor. For example, NDF/Heregulin can stimulate tyrosine phosphorylation of erbB-2 through heterodimerization with either receptors erbB-3 or erbB-4 (Peles *et al.*, 1992, *Cell* **69**, 205-216, Peles *et al.*, 1993, *EMBO J.* **3**, 961-71, Holmes *et al.*,
10 1992, *Science* **256**, 1205-1210; Tzahar *et al.*, 1994, *Biol. Chem.*, **269**, 25226-25233;
Plowman *et al.*, 1993, *Nature* **366**, 473-475; Pinkas-Kramarski *et al.*, 1994, *Proc. Natl. Acad.
Sci. USA*, **91**, 9387-9391; Pinkas-Kramarski *et al.*, 1996, *J. Biol. Chem.*, **271**, 19029-19032;
Pinkas-Kramarski *et al.*, 1998, *Oncogene*, **16**, 1249-1258). Depending on the cell line
15 studied, NDF/Heregulin can either elicit a growth arrest and differentiation phenotype,
resulting in morphological changes, induction of lipids, and expression of intracellular
adhesion molecule-1; or it can induce a mitogenic response (Holmes *et al.*, 1992, *Science*,
256:1205-1210; Peles *et al.*, 1992, *Cell*, 69:205-216; Bacus *et al.*, 1993, *Cancer Res.*
53:5251-5261).

Downstream signaling after ligand binding may be determined by the set of docking proteins that may bind to the activated receptors. For example, activation of erbB receptor
20 heterodimers is coupled to and stimulates downstream MAPK-Erk1/2 and PI3K-AKT growth and survival pathways, whose deregulation in cancer has been linked to disease progression and refractoriness to therapy (Tzahar *et al.*, 1996, *Mol. Cell. Biol.* **16**, 5276-5287; Fukazawa *et al.*, 1996, *J. Biol. Chem.* **271**, 14554-14559, Olayioye *et al.*, 1998, *Mol. Cell. Biol.* **18**, 5042-5051; Lange *et al.*, 1998, *J. Biol. Chem.* **273**, 31308-31316; Hackel *et al.*, 1999, *Curr.
25 Opin. Cell Biol.* **11**, 184-189). HER-3 is a major docking site for phosphoinositide-3-kinase (PI3K). In addition, NDF/Heregulin stimulation causes activation of the PI3K pathway and phosphorylation of AKT (Altikok *et al.*, 1999, *J. Bio. Chem.* **274**, 32274-32278;; Liu *et al.*, 1999, *Biochem. Biophys. Res. Comm.* **261** 897-903; Xing *et al.*, 2000, *Nature, Med.* **6** 189-195). These observations implicate PI3K/AKT in the signaling cascade that results from
30 HER-3 heterodimerization with overexpressed HER-2/neu receptors in breast cancer cells; activation of PI3K/AKT promotes cell survival and enhanced tumor aggressiveness (Bacus *et*

al., 2002, *Oncogene* **21**, 3532-3540). In addition, AKT2 was reported to be activated and overexpressed in HER-2/neu-overexpressing breast cancers (*Id.*).

erbB-2/HER-2 is overexpressed in 20 to 30% of all breast cancers, and its overexpression is associated with poor prognosis, suggesting that it could be used as a target 5 for anti-tumor agents (Slamon *et al.*, 1987; Hudziak *et al.*, 1989; Tagliabue *et al.*, 1991). In erbB-2-overexpressing breast cancer cells, treatment with antibodies specific to HER-2/erbB-2 in combination with chemotherapeutic agents (such as cisplatin, doxubicin, and taxol) elicits a higher cytotoxic response than treatment with chemotherapy alone (Hancock *et al.*, 1991; Arteaga *et al.*, 1994; Pietras *et al.*, 1994). One possible mechanism by which HER-10 2/erbB-2 antibodies might enhance cytotoxicity to chemotherapeutic agents is through the modulation of the HER-2/erbB-2 protein expression (Bacus *et al.*, 1992 & 1993; Stancovski *et al.*, 1991; Klapper *et al.*, 1997 & 2000), or by interfering with DNA repair (Arteaga *et al.*, 1994 & 2001; Pietras *et al.*, 1994).

Because of the effect of anti-HER-2/erbB-2 antibodies on cellular growth, a number 15 of approaches have been used to therapeutically target HER-2/erbB-2- or EGFR-overexpressing cancers. For clinical use, one approach is to interfere with the kinase activity of the receptor by using inhibitors that block the nucleotide binding site of HER-2/neu or EGFR (Bruns *et al.*, 2000; Christensen *et al.*, 2001, Erlichman *et al.*, 2001, Herbst *et al.*, 2002; Hidalgo *et al.*, 2001; Moasser *et al.*, 2001; Fujimura *et al.*, 2002; Normanno *et al.*, 2002). A second approach is using ansamycins to influence the stability of HER2/neu receptors (Munster *et al.*, 2002; Basso *et al.*, 2002). Another approach is the use of antibodies directed to various erbB receptors, specifically EGFR or HER-2/neu (Alaoui-Jamali *et al.*, 1997; Albanell *et al.*, 2001(a); Baselga *et al.*, 1994 & 2002; Mendelsohn, 1990). Analysis of various antibodies to HER-2/neu led to the identification of the murine monoclonal, 4D5. 20 25 This antibody recognizes an extracellular epitope (amino acids 529 to 627) in the cysteine-rich II domain that resides very close to the transmembrane region. Treatment of breast cancer cells with 4D5 partially blocks NDF/hereregulin activation of HER-2-HER-3 complexes, as measured by receptor phosphorylation assays. To allow for chronic human administration, murine 4D5 was fully humanized to generate trastuzumab/HERCEPTIN® 30 (Sliwkowski *et al.*, 1999; Ye *et al.*, 1999; Carter *et al.*, 1992; Fujimoto-Ouchi *et al.*, 2002; Vogel, *et al.*, 2001 & 2002).

A number of monoclonal antibodies and small molecule, tyrosine kinase inhibitors targeting EGFR or erbB-2 have been developed. For example, HERCEPTIN® is approved for treating the 25% of women whose breast cancers overexpress erbB-2 protein or demonstrate erbB-2 gene amplification (Cobleigh *et al.*, 1999, *J. Clin. Oncol.* 17, 2639-2648). In 5 addition, several EGFR-targeted therapies are currently under clinical investigation (Mendelsohn & Baselga, 2000, *Oncogene* 19, 6550-6565; Xia *et al.*, 2002, *Oncogene* 21, 6255-6263).

The development of successful oncological drugs has followed a well-established evaluation process including phases I, II, and III clinical trial. Phase I studies aim to determine the maximally tolerated dose of the drug, its optimal schedule of administration 10 and the dose-limiting toxicities. Historically, cytotoxic cancer therapies have been developed based on maximum tolerated doses (MTD), treating patients without understanding the tumor profile for likely responders. Hence, patients were often subjected to toxic therapies with limited therapeutic benefit. Recently, elucidating tumor growth and survival pathways has led to the development of tumor-targeted therapies. For such targeted therapeutics that are 15 not expected to produce serious adverse side effects, relying on a MTD may not be suitable. More relevant may be the determination of the optimal dose and schedule that is sufficient to inhibit cellular signaling in patient samples. Biological assays for signaling biomarkers are needed to establish such a protocol.

Preclinically, most erbB-receptor targeted therapies primarily exert cytostatic anti- 20 tumor effects, necessitating their chronic administration in clinical practice. Identification of biologically effective doses (BED), the dose or dose range that maximally inhibits the intended target, beyond which dose escalation is likely to add toxicity without benefit, is therefore essential. Moreover, many of these agents will be used in combination with cytotoxic therapies, where added toxicity may not be tolerable, further supporting BED- 25 based dosing. “Targeted –therapy” implies that populations of likely responders exists, and can be identified.

In view of the severe and deleterious consequences of administering an inappropriate or ineffective therapy to a human cancer patient, there exists a need in the art for predicting the response to cancer therapy in an individual. Further, there is a need to develop 30 diagnostics that are capable of predicting patient response for the successful development and clinical acceptance of new HER-2 targeted therapeutics similar to HERCEPTIN®.

SUMMARY OF THE INVENTION

This invention provides methods for predicting a response of an individual to a
5 HER2-directed therapy.

In a first aspect, the invention provides methods for identifying a mammalian tumor
that responds to a HER2-directed therapy, wherein the mammalian tumor overexpresses
HER2, the method comprising the step of assaying a sample obtained from the mammalian
tumor to detect a pattern of expression, phosphorylation or both expression and
10 phosphorylation of one or a plurality of polypeptides consisting of:

- (a) IGFR polypeptide;
- (b) EGFR polypeptide;
- (c) NDF polypeptide;
- (d) phosphorylated S6 ribosomal polypeptide;
- 15 (e) phosphorylated AKT polypeptide; and
- (f) phosphorylated ERK polypeptide;

wherein the particular combination of polypeptides and pattern of expression,
phosphorylation or both expression and phosphorylation identifies mammalian tumors that
respond to a HER2-directed therapy.

20 In certain embodiments, the pattern that identifies a mammalian tumor as responding
is decreased expression of IGFR polypeptide in the mammalian tumor as compared to a non-
tumor tissue or cell sample. In other embodiments, the detected pattern is normal or
increased expression of IGFR polypeptide, accompanied by decreased phosphorylation of
AKT polypeptide, decreased phosphorylation of S6 ribosomal polypeptide or both in the
25 mammalian tumor as compared to a non-tumor tissue or cell sample. In further
embodiments, the detected pattern is normal or increased expression of EGFR polypeptide,
accompanied by decreased phosphorylation of ERK polypeptide in the mammalian tumor as
compared to a non-tumor tissue or cell sample. In additional embodiments, the detected
pattern is decreased expression of IGFR polypeptide, accompanied by increased
30 phosphorylation of S6 ribosomal polypeptide in the mammalian tumor as compared to a non-
tumor tissue or cell sample. In other embodiments, the detected pattern is decreased

expression of IGFR polypeptide, accompanied by increased expression of NDF polypeptide in the mammalian tumor as compared to a non-tumor tissue or cell sample; where further the detected pattern can include increased phosphorylation of S6 ribosomal polypeptide.

In a second aspect, the invention provides methods for identifying a mammalian tumor that does not respond to a HER2-directed therapy, wherein the mammalian tumor overexpresses HER2, the method comprising the step of assaying a sample obtained from the mammalian tumor to detect a pattern of expression, phosphorylation or both expression and phosphorylation of one or a plurality of polypeptides consisting of:

- (a) IGFR polypeptide;
- (b) EGFR polypeptide;
- (c) NDF polypeptide;
- (d) phosphorylated S6 ribosomal polypeptide;
- (e) phosphorylated AKT polypeptide; and
- (f) phosphorylated ERK polypeptide;

wherein the particular combination of polypeptides and pattern of expression, phosphorylation or both expression and phosphorylation identifies mammalian tumors that do not respond to a HER2-directed therapy.

In certain embodiments, the pattern that identifies a mammalian tumor as not responding is normal or increased expression of IGFR polypeptide, accompanied by increased phosphorylation of AKT polypeptide, increased phosphorylation of S6 ribosomal polypeptide, or both in the mammalian tumor as compared to a non-tumor tissue or cell sample. In other embodiments, the detected pattern is decreased expression of EGFR polypeptide and increased expression of NDF polypeptide in the mammalian tumor as compared to a non-tumor tissue or cell sample. In further embodiments, the detected pattern is decreased expression of EGFR polypeptide in the mammalian tumor as compared to a non-tumor tissue or cell sample. In other embodiments, the detected pattern is decreased expression of NDF polypeptide in the mammalian tumor as compared to a non-tumor tissue or cell sample. In additional embodiments, the detected pattern is decreased expression of EGFR polypeptide and increased phosphorylation of ERK polypeptide in the mammalian tumor as compared to a non-tumor tissue or cell sample. In further embodiments, the detected pattern is normal or increased expression of IGFR polypeptide and decreased

expression of NDF in the mammalian tumor as compared to a non-tumor tissue or cell sample.

In a third aspect, the invention provides methods of selecting a subject with cancer for treatment with a molecule targeting HER2, wherein the cancer overexpresses HER2, the

5 methods comprising the steps of:

(a) determining the pattern of expression, phosphorylation or both expression and phosphorylation in a cell or tissue sample from the subject of one or a plurality of polypeptides consisting of:

- (i) IGFR polypeptide;
- (ii) EGFR polypeptide;
- (iii) NDF polypeptide;
- (iv) phosphorylated S6 ribosomal polypeptide;
- (v) phosphorylated AKT polypeptide; and
- (vi) phosphorylated ERK polypeptide; and

15 (b) selecting the subject based on the detected pattern of expression, phosphorylation, or both expression and phosphorylation. The particular combination of polypeptides and pattern of expression, phosphorylation or both expression and phosphorylation is used to select the subjects with cancer for treatment with a molecule targeting HER2.

20 In certain embodiments, the detected pattern for selecting a subject for treatment with a molecule targeting HER2 is decreased expression of IGFR polypeptide in the mammalian tumor as compared to a non-tumor tissue or cell sample. In other embodiments, the detected pattern is normal or increased expression of IGFR polypeptide, accompanied by decreased phosphorylation of AKT polypeptide, decreased phosphorylation of S6 ribosomal polypeptide or both in the mammalian tumor as compared to a non-tumor tissue or cell sample. In further embodiments, the detected pattern is normal or increased expression of EGFR polypeptide, accompanied by decreased phosphorylation of ERK polypeptide in the mammalian tumor as compared to a non-tumor tissue or cell sample. In additional embodiments, the detected pattern is decreased expression of IGFR polypeptide, accompanied by increased phosphorylation of S6 ribosomal polypeptide in the mammalian tumor as compared to a non-tumor tissue or cell sample. In other embodiments, the detected

pattern is decreased expression of IGFR polypeptide, accompanied by increased expression of NDF polypeptide in the mammalian tumor as compared to a non-tumor tissue or cell sample; where further the detected pattern can include increased phosphorylation of S6 ribosomal polypeptide.

5 In a fourth aspect, the invention provides methods of selecting a subject with cancer to not receive treatment with a molecule targeting HER2, wherein the cancer overexpresses HER2, the methods comprising the steps of:

(a) determining the pattern of expression, phosphorylation or both expression and phosphorylation in a cell or tissue sample from the subject of one or a plurality of

10 polypeptides consisting of:

- (i) IGFR polypeptide;
- (ii) EGFR polypeptide;
- (iii) NDF polypeptide;
- (iv) phosphorylated S6 ribosomal polypeptide;
- (v) phosphorylated AKT polypeptide; and
- (vi) phosphorylated ERK polypeptide; and

15 (b) selecting the subject based on the detected pattern of expression, phosphorylation, or both expression and phosphorylation. The particular combination of polypeptides and pattern of expression, phosphorylation or both expression and phosphorylation is used to select the subjects with cancer to not receive treatment with a molecule targeting HER2.

20 In certain embodiments, the detected pattern for selecting a subject not to receive treatment with a molecule targeting HER2 is normal or increased expression of IGFR polypeptide, accompanied by increased phosphorylation of AKT polypeptide, increased phosphorylation of S6 ribosomal polypeptide, or both in the mammalian tumor as compared to a non-tumor tissue or cell sample. In other embodiments, the detected pattern is decreased expression of EGFR polypeptide and increased expression of NDF polypeptide in the mammalian tumor as compared to a non-tumor tissue or cell sample. In further embodiments, the detected pattern is decreased expression of EGFR polypeptide in the mammalian tumor as compared to a non-tumor tissue or cell sample. In other embodiments, 25 the detected pattern is decreased expression of NDF polypeptide in the mammalian tumor as

compared to a non-tumor tissue or cell sample. In additional embodiments, the detected pattern is decreased expression of EGFR polypeptide and increased phosphorylation of ERK polypeptide in the mammalian tumor as compared to a non-tumor tissue or cell sample. In further embodiments, the detected pattern is normal or increased expression of IGFR 5 polypeptide and decreased expression of NDF in the mammalian tumor as compared to a non-tumor tissue or cell sample.

In various aspects of the invention, including those mentioned above, the detection of phosphorylation of AKT polypeptide, phosphorylation of S6 ribosomal polypeptide, or both can be determined subsequent to contacting the sample obtained from the mammalian tumor 10 with a HER2-directed therapy. Further, the HER2-directed therapy can be or comprise rhuMAb HER2 (HERCEPTIN®). In addition, the sample can be contacted with at least one chemotherapeutic agent. Further, the detected pattern of expression, phosphorylation, or both, of one or a plurality of polypeptides (a) through (f) can be determined using a biodetection reagent. The biodetection reagent can be an antibody or a nucleic acid probe. 15 Further, the detected pattern of phosphorylated AKT polypeptide can be determined using an antibody specific for an epitope comprising a phosphorylated serine residue at position 473, the detected pattern of phosphorylated S6 ribosomal polypeptide can be determined using an antibody specific for an epitope comprising a phosphorylated serine residue at position 235, and/or the detected pattern of phosphorylated ERK polypeptide can be determined using an 20 antibody specific for an epitope comprising a phosphorylated threonine residue at position 202 and a phosphorylated tyrosine residue at position 204. Further, the sample obtained from the mammalian tumor can be a paraffin-embedded biopsy sample. Also, the mammalian tumor can be identified as overexpressing HER2 using an antibody that binds HER2 polypeptide.

25 In a fifth embodiment, the invention provides kits for characterizing a mammalian tumor's responsiveness to a HER2-directed therapy, the kit comprising:

- (a) an antibody that binds IGFR polypeptide,
- and one or more of the following:
- (b) an antibody that binds phosphorylated AKT polypeptide;
- (c) an antibody that binds phosphorylated S6 ribosomal polypeptide;
- (d) an antibody that binds EGFR polypeptide;

- (e) an antibody that binds HER2 polypeptide;
- (f) an antibody that binds NDF polypeptide; and
- (g) an antibody that binds phosphorylated ERK polypeptide.

In certain embodiments, the antibody of (b) is immunologically specific for AKT polypeptide
5 having a phosphorylated serine residue at position 473; antibody of (c) is immunologically
specific for S6 ribosomal polypeptide having a phosphorylated serine residue at position 235;
and/or the antibody of (f) is immunologically specific for EKT polypeptide having a
phosphorylated threonine residue at position 202 and a phosphorylated tyrosine at position
204. In other embodiments, the kit further comprises at least one secondary antibody that
10 binds to an antibody of subpart (a) through (g).

Specific embodiments of the present invention will become evident from the
following more detailed description of certain preferred embodiments and the claims.

15 **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

This invention provides methods for predicting response in cancer subjects to cancer
therapy, including human cancer patients. In addition, this invention provides predictive
biomarkers to identify the cancer patients for whom the administering a therapeutic agent
will be most effective, including a therapeutic agent for treating breast cancer. Specifically,
20 this invention provides predictive biomarkers for assessing the efficacy of therapeutic agents
targeted to Her2/neu, including such agents such as HERCEPTIN®.

In contrast to traditional anticancer methods, where chemotherapeutic drug treatment
is undertaken as an adjunct to and after surgical intervention, neoadjuvant (or primary)
chemotherapy consists of administering drugs as an initial treatment in certain cancer
25 patients. One advantage of such an approach is that, for primary tumors of more than 3 cm,
it permits the later or concomitant use of conservative surgical procedures (as opposed to,
e.g., radical mastectomy in breast cancer patients) for the majority of patients, due to the
tumor shrinking effect of the chemotherapy. Another advantage is that for many cancers, a
partial and/or complete response is achieved in about two-thirds of all patients. Finally,
30 because the majority of patients are responsive after two to three cycles of chemotherapeutic
treatment, it is possible to monitor the *in vivo* efficacy of the chemotherapeutic regimen

employed, in order to identify patients whose tumors are non-responsive to chemotherapeutic treatment. Timely identification of non-responsive tumors allows the clinician to limit a cancer patient's exposure to unnecessary side-effects of treatment and to institute alternative treatments. Unfortunately, methods present in the art, including histological examination, are 5 insufficient for such a timely and accurate identification. The present invention provides methods for developing more informed and effective regimes of therapy that can be administered to cancer patients with an increased likelihood of an effective outcome (i.e., reduction or elimination of the tumor).

A cancer diagnosis, both an initial diagnosis of disease and subsequent monitoring of 10 the disease course (before, during, or after treatment) is conventionally confirmed through histological examination of cell or tissue samples removed from a patient. Clinical pathologists need to be able to accurately determine whether such samples are benign or malignant and to classify the aggressiveness of tumor samples deemed to be malignant, because these determinations often form the basis for selecting a suitable course of patient 15 treatment. Similarly, the pathologist needs to be able to detect the extent to which a cancer has grown or gone into remission, particularly as a result of or consequent to treatment, most particularly treatment with chemotherapeutic or biological agents.

Histological examination traditionally entails tissue-staining procedures that permit 20 morphological features of a sample to be readily observed under a light microscope. A pathologist, after examining the stained sample, typically makes a qualitative determination of whether the tumor sample is malignant. It is difficult, however, to ascertain a tumor's aggressiveness merely through histological examination of the sample, because a tumor's aggressiveness is often a result of the biochemistry of the cells within the tumor, such as protein expression or suppression and protein phosphorylation, which may or may not be 25 reflected by the morphology of the sample. Therefore, it is important to be able to assess the biochemistry of the cells within a tumor sample. Further, it is desirable to be able to observe and quantitate both gene expression and protein phosphorylation of tumor-related genes or proteins, or more specifically cellular components of tumor-related signaling pathways.

Cancer therapy can be based on molecular profiling of tumors rather than simply their 30 histology or site of the disease. Elucidating the biological effects of targeted therapies in tumor tissue and correlating these effects with clinical response helps identify the

predominant growth and survival pathways operative in tumors, thereby establishing a pattern of likely responders and conversely providing a rational for designing strategies to overcome resistance. Successful diagnostic targeting of a growth factor receptor must determine if tumor growth or survival is being driven by the targeted receptor or receptor family, by other receptors not targeted by the therapy, and whether downstream signaling suggests that another oncogenic pathway is involved.

For subjects considered for treatment with HERCEPTIN®, it is necessary to consider additional biomarkers beyond the presence of the target HER-2/neu, at least because the status of the EGFR and erbB ligands NDF and TGF- α affect HERCEPTIN® therapy response in breast cancer patients. Therefore, considering HER2/neu expression alone does not necessarily predict overall erbB oncogenic activity or potential response to erbB inhibitors. In addition, previous studies have shown that not all tumor cells respond to inhibition of ErbB receptors, despite exhibiting aberrant EGFR and/or HER2/neu expression. Examples include the MKN7 and BT474 erbB receptor-overexpressing carcinoma cell lines: BT474 cells respond to HERCEPTIN® but MKN7 cells do not (Motoyama, *et al.*, *Cancer Research*, **62**, 3151-3158 (2002)). In addition, the proliferation block induced as a consequence of decreased EGFR or HER2 receptor activity, such as by the activity of an erbB-inhibitor, may be overcome by the presence of EGF-related ligands such as EGF or NDF/Heregulin (*Id*). This phenomenon can be attenuated using a bispecific ErbB-1/ErbB-2 inhibitor, thus supporting increased antitumor efficacy through simultaneous inhibition of multiple ErbB receptors.

In addition, in many cancers NDF/Heregulin or TGF- α expression produces an autocrine loop of HER-2/EGFR heterodimerization. Downregulation of HER-2/neu expression is an important way of inhibiting signals generated by these heterodimers. Downregulation can be accomplished by treatment with HERCEPTIN®, which produces receptor endocytosis. Furthermore, high levels of phosphorylated ERK (or pAKT) can be a negative predictor for positive treatment outcome, when observed in conjunction with the expression of EGFR and the presence of NDF, suggesting the existence of other pathways that might promote proliferation of the tumor cellular growth. High pERK is also associated with resistance to HERCEPTIN® through downregulation of p27; this may implicate other signals (such as estrogen receptor's cross activation of the MAPK and AKT pathways) that

may contribute to high pERK and thus contribute to proliferation of the tumor cells growth. In addition, phosphorylated AKT has been shown to be an important part of the response to HERCEPTIN®, as high pAKT-positive patients had poor response to HERCEPTIN®. High-phosphorylated AKT has been shown to be associated with high expression of insulin like growth factor receptors (IGFR-1) as well as PDGFR and results in resistance to HERCEPTIN®. Interestingly, data from clinical trials have shown that using a dual inhibitor (*i.e.*, specific for HER-1/neu and HER-2/neu) has clinical efficacy in patients when treatment induced downregulation of pERK and pAKT, but not in patients in which pERK and pAKT levels didn't diminish after treatment. Thus, in those patients who overexpressed 5 HER-1 and HER-2, as well as pERK and pAKT, antitumor activity was dependent on HER-1 and HER-2 receptor activation and a clinical response was observed. In contrast, in patients for whom pERK and pAKT activity remained high after treatment with a dual inhibitor, clinical response didn't occur. Combination therapies can have clinical significance. A combination of the ErbB-1-directed monoclonal antibodies mAb 225 and mAb 4D5 inhibited 10 proliferation of an ovarian tumor cell line more strongly than either mAb alone (Ye *et al.*, 1999, *Oncogene* 18: 731-8). In addition to ErbB-targeted mAbs, a number of different ErbB-1/ErbB-2-bispecific inhibitors, also referred to as dual EGFR/erbB-2 kinase inhibitors, have been described recently, such as GW572016 and PKI166, that are currently in clinical trials 15 (Motoyama *et al.*, 2002, *Cancer Research* 62: 3151-3158). Therefore, response to HERCEPTIN® is affected by the expression of multiple erbB receptors and their ligands in tumors.

Thus, HER-2/neu overexpression alone is not the only predictor of response to molecules such as HERCEPTIN®. HER-2/neu is an orphan, ligandless receptor in need of its partners EGFR (HER-1) and HER-3 in order to exert its activity. HER-1 and HER-3 20 heterodimerization with HER-2 is enhanced by the presence of EGF or NDF (Tzahar *et al.*, 1996, *EMBO J.* 15: 254-64, Graus-Porta, 1997, *EMBO J.*, 16 1647-55), and thus HER-2 activity is dependant on HER-1 or HER-3. Other receptors may also transactivate the erbB receptors. These receptors may be mediating tumorigenesis through signaling to downstream 25 proliferative and survival pathways. For example, the IGFR receptor may mediate patient response to breast cancer therapies targeting HER2/neu. High IGFR expression combined with high S6 ribosomal protein phosphorylation correlates with poor patient response 30

regardless of erb-B expression, indicating that IGFR acts directly to activate signaling downstream of erb-B receptors rather than through transactivation of erb-B receptors. Cell line studies also have suggested a role for IGFR in patient response. HERCEPTIN® resistance has been suggested to occur though activation of IGFR (Lu *et al.*, 2001, *J. National Cancer Institute* **93**: 1852). In addition, co-targeting IGFR as well as HER2/neu has been shown to produce synergistic inhibition of growth in breast cancer cells (Camirand *et al.*, 2002, *Med Sci Monit.* **8**: (12): BR521-6). Therefore, analysis of IGFR expression and downstream signaling can be critical for an accurate assessment of potential HERCEPTIN® response in breast cancer patients.

Thus, there is no one marker of downstream signaling protein activation that would integrate multiple upstream signals and predict response. However, analysis of p-ERK and p-AKT has been found to be predictive in patients over-expressing EGFR. Therefore, in the presence of active erbB receptors, high ERK and AKT signaling indicates that HERCEPTIN® therapy is less likely to be effective. AKT activation has been shown to result in HERCEPTIN® resistance in breast cancer cell lines (Yakes, *et al.*, 2002, *Cancer Res.* **62**: 4132-41; Clark *et al.*, 2002, *Mol. Cancer Ther.* **1**: 707-17). In addition, analysis of S6 ribosomal protein phosphorylation greatly increased the predictive power of IGFR expression. In patients with high S6 phosphorylation, positive response ranged from 8% to 67% based upon IGFR expression. Approximately 30% of patients with low S6 phosphorylation responded, regardless of IGFR expression. These results were also reflected in an analysis of clinical samples, in which only those patients that lacked active IGFR signaling responded to HERCEPTIN® therapy. IGF signaling in breast cancer occurs through AKT activation (Oh *et al.*, 2002, *Neoplasia* **4**: 204-17; Dufourny *et al.*, 1997, *J Biol Chem.* **272**: 31163-71), which leads to S6 ribosomal protein phosphorylation. Hence, S6 phosphorylation can be indicative of active IGF signaling in those tumors over-expressing IGFR.

Analysis of down-stream signaling and patient response is complicated when chemotherapy and radiotherapy therapy is included in addition to HERCEPTIN® treatment. AKT and MAP kinase pathway activation, for example, are known to play a role in response to DNA-damaging agents (Clark *et al.*, 2002, *Mol. Cancer Ther.* **1**: 707-17; Bacus *et al.*, 2001, *Oncogene* **20**: 147-155). Consideration of downstream signaling in patients

undergoing a combination of therapies provides additional predictive information not obtained solely from analysis of receptor or ligand expression levels. Analysis of patients treated with HERCEPTIN® as a single agent therapy can be used to determine which of the identified biomarkers mediated the response to Herceptin,® as opposed to the biomarkers that 5 mediate the response to the other therapies. Nonetheless, the identified biomarkers are useful, among other things, for designing diagnostics for breast cancer patients undergoing the common HERCEPTIN® combination therapies.

Further, up-regulation of the AKT/mTOR pathway by Heregulin/NDF is an important predictor for response. pAkt has been associated with high levels of Cyclin E and low 10 levels of the cyclin inhibitor p27.

Before administration of HER2-targeted therapies, a panel of diagnostics of each tumor is used according to the methods of this invention to find the best candidate for each therapy. According to the methods of this invention, treatment by a HER2-targeted therapy, such as HERCEPTIN®, is effective when a patient's tumor growth depends on a cellular 15 pathway such as AKT/mTOR that is driven by the erbB receptors and not by other tyrosine kinases, such as Insulin-like Growth Factor Receptors (IGFR). When high levels of activation of these downstream signals occur independent of erbB receptors, HERCEPTIN® treatment is not effective. Use of the methods of this invention permits a clinician to choose a more effective combination of targeted therapies for cancer patients.

20 The HER2-directed therapies of the present invention can include, for example, rhuMAb HER2, otherwise known as HERCEPTIN®. The samples obtained from the mammalian tumor can be contacted with at least one chemotherapeutic agent, for example cisplatin, doxorubicin, or taxol.

Automated (computer-aided) image analysis systems known in the art can augment 25 visual examination of tumor samples. In a representative embodiment, the cell or tissue sample is exposed to detectably-labeled reagents specific for a particular biological marker, and the magnified image of the cell is then processed by a computer that receives the image from a charge-coupled device (CCD) or camera such as a television camera. Such a system can be used, for example, to detect and measure expression and activation levels of EGFR, 30 HER2, HER3, pERK, NDF, TGF- α , IGFR, pS6, and pAkt in a sample, or any additional diagnostic biomarkers. Thus, the methods of the invention provide more accurate cancer

diagnosis and better characterization of gene expression in histologically identified cancer cells, most particularly with regard to expression of tumor marker genes or genes known to be expressed in particular cancer types and subtypes (e.g., having different degrees of malignancy). This information permits a more informed and effective regimen of therapy to
5 be administered, because drugs with clinical efficacy for certain tumor types or subtypes can be administered to patients whose cells are so identified.

Another drawback of conventional anticancer therapies is that the efficacy of specific chemotherapeutic agents in treating a particular cancer in an individual human patient is unpredictable. In view of this unpredictability, the art is unable to determine, prior to starting
10 therapy, whether one or more selected agents would be active as anti-tumor agents or to render an accurate prognosis of course of treatment in an individual patient. This is especially important because a particular clinical cancer may present the clinician with a choice of treatment regimens, without any current way of assessing which regimen will be most efficacious for a particular individual. It is an advantage of the methods of this
15 invention that they are able to better assess the expected efficacy of a proposed therapeutic agent (or combination of agents) in an individual patient. The claimed methods are advantageous for the additional reasons that they are both time- and cost-effective in assessing the efficacy of chemotherapeutic regimens and are minimally traumatic to cancer patients.

20 Methods of this invention can be used to identify a mammalian tumor that responds to HER-2 directed therapies. Further, methods of this invention can be used to select a subject with cancer for treatment with a molecule targeting HER. Moreover, methods of this invention can be used to identify a mammalian tumor that does not respond to HER-2 directed therapies. Further, methods of this invention can be used to select a subject with
25 cancer to not receive treatment with a molecule targeting HER2.

Patterns of expression and phosphorylation of polypeptides are detected and quantified using methods of the present invention. More particularly, patterns of expression and phosphorylation of polypeptides that are cellular components of a tumor-related signaling pathway are detected and quantified using methods of the present invention. For
30 example, the patterns of expression and phosphorylation of polypeptides can be detected using biodetection reagents specific for the polypeptides. For example, the biodetection

reagents can be antibodies. Alternatively, the biodetection reagents can be nucleic acid probes. A nucleic acid probe is defined to be a collection of one or more nucleic acid fragments whose hybridization to a sample can be detected. The probe may be unlabeled or labeled so that its binding to the target or sample can be detected. The probe is produced
5 from a source of nucleic acids from one or more particular (preselected) portions of the genome, e.g., one or more clones, an isolated whole chromosome or chromosome fragment, or a collection of polymerase chain reaction (PCR) amplification products. The nucleic acid probe may also be isolated nucleic acids immobilized on a solid surface (e.g., nitrocellulose, glass, quartz, fused silica slides), as in an array. The probe may be a member of an array of
10 nucleic acids as described, for instance, in WO 96/17958. Techniques capable of producing high density arrays can also be used for this purpose (see, e.g., Fodor (1991) Science 767-773; Johnston (1998) Curr. Biol. 8: R171-R174; Schummer (1997) Biotechniques 23: 1087-1092; Kern (1997) Biotechniques 23: 120-124; U.S. Pat. No. 5,143,854). One of skill will recognize that the precise sequence of the particular probes can be modified to a certain
15 degree to produce probes that are "substantially identical," but retain the ability to specifically bind to (i.e., hybridize specifically to) the same targets or samples as the probe from which they were derived. The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide in either single- or double-stranded form. The term encompasses nucleic acids, i.e., oligonucleotides, containing known analogues of natural nucleotides that have
20 similar or improved binding properties, for the purposes desired, as the reference nucleic acid. The term also includes nucleic acids which are metabolized in a manner similar to naturally occurring nucleotides or at rates that are improved for the purposes desired. The term also encompasses nucleic-acid-like structures with synthetic backbones. One of skill in the art would recognize how to use a nucleic acid probes for screening of cancer cells in a
25 sample by reference, for example, to U.S. Patent 6,326,148, directed to screening of colon carcinoma cells.

Polypeptides associated with breast cancer can be quantified by image analysis using a suitable primary antibody against biomarkers, including but not limited to EGFR, HER-2, HER-3, IGFR, NDF, TGF- α , p-ERK, pS6, or p-AKT, detected directly or using an
30 appropriate secondary antibody (such as rabbit anti-mouse IgG when using mouse primary antibodies) and/or a tertiary avidin (or Streptavidin) biotin complex ("ABC").

Examples of reagents useful in the practice of the methods of the invention as exemplified herein include antibodies specific for HER2/neu, including but not limited to the mouse monoclonal antibody CB11, obtained from Ventana Medical Systems, Inc. (VMSI, Tucson, AZ). In addition, reagents useful in the practice of the methods of the invention 5 include antibodies specific for phosphorylated AKT, including but not limited to antibodies specific for a phosphorylated serine residue of position 473, wherein the sequence of AKT is represented by SEQ ID NO:1 (Table 8). Further, reagents useful in the practice of the methods of the invention include antibodies specific for phosphorylated S6, including but not limited to antibodies specific for a phosphorylated serine residue of position 235, wherein the 10 sequence of S6 is represented by SEQ ID NO:2 (Table 8). Also, reagents useful in the practice of the methods of the invention include antibodies specific for phosphorylated ERK, including but not limited to antibodies specific for a phosphorylated threonine residue at position 202 and a phosphorylated tyrosine residue of position 204, wherein the sequence of ERK is represented by SEQ ID NO:3 (Table 8).

15 Further, the pattern of expression, phosphorylation, or both expression and phosphorylation of the predictive polypeptides can be compared to a non-tumor tissue or cell sample. The non-tumor tissue or cell sample can be obtained from a non-tumor tissue or cell sample from the same individual, or alternatively, a non-tumor tissue or cell sample from a different individual. A detected pattern for a polypeptide is referred to as decreased in the 20 mammalian tumor, tissue, or cell sample, if there is less polypeptide detected as compared to the a non-tumor tissue or cell sample. A detected pattern for a polypeptide is referred to as increased in the mammalian tumor, tissue, or cell sample, if there is more polypeptide detected as compared to the a non-tumor tissue or cell sample. A detected pattern for a polypeptide is referred to as normal in the mammalian tumor, tissue, or cell sample, if there 25 is the same, or approximately the same, polypeptide detected as compared to the a non-tumor tissue or cell sample.

The methods of this invention for identifying mammalian tumors that respond, or that do not respond, to a HER2-directed therapy comprise the step of assaying a sample obtained from the mammalian tumor to detect a pattern of expression, phosphorylation or both of one 30 or a plurality of polypeptides consisting of: (a) IGFR polypeptide; (b) EGFR polypeptide; (c) NDF polypeptide; (d) phosphorylated S6 ribosomal polypeptide; (e) phosphorylated AKT

polypeptide; (f) phosphorylated EKT polypeptide. The combination of polypeptides and pattern of expression, phosphorylation, or both expression and phosphorylation identifies mammalian tumors that respond, or that do not respond, to a HER2-directed therapy. The methods can include the detection of a pattern of expression, phosphorylation or both of one, 5 two, three, four, five, or all six of these polypeptides. Further, the methods can, but need not, include other steps, including steps such as the detection of a pattern of expression, phosphorylation or both of different polypeptides.

The methods of this invention for selecting a subject with cancer for treatment, or to not receive treatment, with a molecule targeting HER2, such as, but not limited to treatment 10 with HERCEPTIN®, comprise the step of determining the pattern of expression, phosphorylation or both in a cell or tissue sample from the subject of one or a plurality of polypeptides consisting of: (a) IGFR polypeptide; (b) EGFR polypeptide; (c) NDF polypeptide; (d) phosphorylated S6 ribosomal polypeptide; (e) phosphorylated AKT polypeptide; (f) phosphorylated EKT polypeptide. The combination of polypeptides and 15 pattern of expression, phosphorylation, or both expression and phosphorylation is used to select a subject with cancer for treatment, or to not receive treatment, with a molecule targeting HER2. The methods can include the detection of a pattern of expression, phosphorylation or both of one, two, three, four, five, or all six of these polypeptides. Further, the methods can, but need not, include other steps, including steps such as the 20 detection of a pattern of expression, phosphorylation or both of different polypeptides.

For example, the pattern that identifies a mammalian tumor as responding or that can be used to select a subject with cancer for treatment with a molecule targeted to HER2 is decreased expression of IGFR polypeptide as compared to a non-tumor tissue or cell sample. Alternatively, the detected pattern is normal or increased expression of IGFR polypeptide, 25 accompanied by decreased phosphorylation of AKT polypeptide, decreased phosphorylation of S6 ribosomal polypeptide or both as compared to a non-tumor tissue or cell sample. Another potential detected pattern is normal or increased expression of EGFR polypeptide, accompanied by decreased phosphorylation of ERK polypeptide as compared to a non-tumor tissue or cell sample. Further detected patterns include decreased expression of IGFR 30 polypeptide, accompanied by increased phosphorylation of S6 ribosomal polypeptide as compared to a non-tumor tissue or cell sample. In other embodiments, the detected pattern is

decreased expression of IGFR polypeptide, accompanied by increased expression of NDF polypeptide in the mammalian tumor as compared to a non-tumor tissue or cell sample; where further the detected pattern can include increased phosphorylation of S6 ribosomal polypeptide. These identified patterns are understood to be non-limiting.

5 For example, the pattern that identifies a mammalian tumor as not responding or that can be used to select a subject with cancer to not receive treatment with a molecule targeted to HER2 is normal or increased expression of IGFR polypeptide, accompanied by increased phosphorylation of AKT polypeptide, increased phosphorylation of S6 ribosomal polypeptide, or both as compared to a non-tumor tissue or cell sample. Alternatively, the
10 detected pattern is decreased expression of EGFR polypeptide and increased expression of NDF polypeptide as compared to a non-tumor tissue or cell sample. Or, the detected pattern is decreased expression of EGFR polypeptide as compared to a non-tumor tissue or cell sample. Further, the detected pattern is decreased expression of NDF polypeptide as
15 compared to a non-tumor tissue or cell sample. Or, the detected pattern is decreased expression of EGFR polypeptide and increased phosphorylation of ERK polypeptide as compared to a non-tumor tissue or cell sample. Further, the detected pattern is normal or increased expression of IGFR polypeptide and decreased expression of NDF as compared to a non-tumor tissue or cell sample. These identified patterns are understood to be non-limiting.

20 In practicing the methods of this invention, staining procedures can be carried out by a person, such as a technician in the laboratory. Alternatively, the staining procedures can be carried out using automated systems. In either case, staining procedures for use according to the methods of this invention are preformed according to standard techniques and protocols well-established in the art.

25 By “cell or tissue sample” is meant biological samples comprising cells, most preferably tumor cells, that are isolated from body samples, such as, but not limited to, smears, sputum, biopsies, secretions, cerebrospinal fluid, bile, blood, lymph fluid, urine and faeces, or tissue which has been removed from organs, such as breast, lung, intestine, skin, cervix, prostate, and stomach. For example, a tissue samples can comprise a region of
30 functionally related cells or adjacent cells.

The amount of target protein is advantageously quantified by measuring the average optical density of the stained antigens. Concomitantly, the proportion or percentage of total tissue area stained can be readily calculated, for example as the area stained above a control level (such as an antibody threshold level) in the second image. Following visualization of 5 nuclei containing biomarkers, the percentage or amount of such cells in tissue derived from patients after treatment are compared to the percentage or amount of such cells in untreated tissue. For purposes of the invention, “determining” a pattern of expression, phosphorylation, or both expression and phosphorylation o polypeptides is understood broadly to mean merely obtaining the information on such polypeptide(s), either through 10 direct examination or indirectly from, for example, a contract diagnostic service.

Breast cancer tissue sections taken from patients treated with HERCEPTIN® and chemotherapy are analyzed, according to the methods of this invention by immunohistochemistry for expression, phosphorylation, or expression and phosphorylation of erb-B ligands, receptors, downstream signaling proteins or any positive treatment response 15 predictive combination thereof. These measurements can be accomplished, for example, by using tissue microarrays. Tissue microarrays are advantageously used in the methods of the invention, being well-validated method to rapidly screen multiple tissue samples under uniform staining and scoring conditions. (Hoos *et al.*, 2001, *Am J Pathol.* **158**: 1245-51). Scoring of the stained arrays can be accomplished by an automated system that accurately 20 quantified the staining observed. The results of this analysis identify biomarkers that best predict patient outcome following treatment, such as HERCEPTIN® therapies. Patient “probability of response” ranging from 0 to 100 percent can be predicted based upon the expression, phosphorylation or both of a small set of ligands, receptors, signaling proteins or predictive combination thereof. Additional samples from breast cancer patients can be 25 analyzed, either as an alternative to or in addition to tissue microarray results. For example, analysis of samples from breast cancer patients can confirm the conclusions from the tissue arrays, if the patient’s responses correlate with a specific pattern of receptor expression and/or downstream signaling.

The invention provides, in part, kits for carrying out the methods of the invention. 30 For example, the method provides kits for characterizing a mammalian tumor’s responsiveness to a HER2-directed therapy comprising an antibody that binds IGFR

polypeptide, and one or more of the following: an antibody that binds phosphorylated AKT polypeptide; an antibody that binds phosphorylated S6 ribosomal polypeptide; an antibody that binds EGFR polypeptide; an antibody that binds HER2 polypeptide; an antibody that binds NDF polypeptide; and an antibody that binds phosphorylated ERK polypeptide. In
5 addition to an antibody that binds IGFR polypeptide, the kit can include one, two, three, four, or all five of the following: an antibody that binds phosphorylated AKT polypeptide; an antibody that binds phosphorylated S6 ribosomal polypeptide; an antibody that binds EGFR polypeptide; an antibody that binds HER2 polypeptide; an antibody that binds NDF polypeptide; and an antibody that binds phosphorylated ERK polypeptide. Further, the kit
10 can include additional components other than the above-identified antibodies, including but not limited to additional antibodies. Such kits may be used, for example, by a clinician or physician as an aid to selecting an appropriate therapy for a particular patient, for example, a breast cancer patient under consideration for HER2-directed therapy.

Particularly useful embodiments of the present invention and the advantages thereof
15 can be understood by referring to Examples 1-5. These Examples are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

20

EXAMPLE 1

Staining procedure for biomarkers

Human tumor tissue sections were stained for predictive biomarkers according to the methods of the invention as follows. 10% Neutral Buffered Formalin Paraffin blocks were sectioned at 4 microns and the sections placed onto coated slides. EGFR and HER2 immunostaining was performed by using the pre-diluted EGFR and HER2 antibodies from Ventana Medical Instruments, Inc. (VMSI, Tucson, AZ.). HER3, Heregulin (NDF), and IGFR antibodies were obtained from NeoMarkers (Fremont, CA.). TGF- α antibodies were obtained from Oncogene Sciences (San Diego, CA). EGFR, HER2/neu, HER3, IGFR, Heregulin, and TGF- α were immunostained using the “BenchMark” (VMSI) with I-VIEW
25 (VMSI) detection chemistry. Antibodies specific for p-ERK (1:100), p-AKT (1:75), and phospho-S6 ribosomal protein were obtained from Cell Signaling Technology (Beverly,
30

MA), and immunostained using a labeled streptavidin peroxidase technique. (Vector Elite ABC Kit, Burlingame, CA). Prior to staining, slides for p-S6 ribosomal protein, p-ERK and p-AKT were antigen retrieved using 0.1 M citrate buffer, pH 6.0 in the “decloaker” (Biocare Corp.) and the sections incubated overnight with the primary antibodies at 4°C. The next 5 day, the slides were placed onto the Autostainer (Dako Corp.) and the “LSAB2” kit (Dako) was employed as the detection chemistry. DAB (Dako) was used as the chromogen. After immunostaining, all slides were counterstained manually with 4% ethyl green (Sigma).

EXAMPLE 2

Procedure for Western Blot Analysis

Western blot analysis for detecting expression of predictive markers was performed as follows. Cells were lysed in ice-cold buffer (50 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1mM EDTA, 1% Nonidet P-40, 0.2% Triton X-100, 10% glycerol, 0.1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 50 mM NaF, 1 15 mM phenylmethylsulfonyl fluoride, 2 µM leupeptin, and 2 µg/ml aprotinin). Protein concentration was determined with a BioRad Protein Assay Kit (BioRad Laboratories, Hercules, CA). Equal amounts of protein, typically 15 µg protein per lane, were separated by gel electrophoresis, for example using pre-cast 4-12% Bis-Tris NuPage gradient gels (Invitrogen) or 7.5% or 4-15% gradient SDS-PAGE under reducing conditions, and 20 transferred to membranes, such as HyBond-C nitrocellulose (Amersham Life Science) or Immobilon-P membranes. Membranes were blocked and then incubated with primary antibodies, for example antibodies against p-AKT and p-ERK (Cell Signaling Technology). Antibody incubation was performed overnight at 4°C in Tris-buffered saline containing 3% bovine serum albumin/0.1% Tween 20. Signal was detected by chemiluminescence 25 (PerkinElmer Life Sciences), or using a SuperSignal West Femto Maximum sensitivity substrate kit from Pierce (Rockford, IL) as described (Xia *et al.*, 2002, *Oncogene* 21: 6255-6263).

EXAMPLE 3

Procedure for Immunohistochemistry

Immunohistochemistry for detecting and measuring predictive biomarker expression, activation or both was performed as follows. HER2/neu, EGFR, HER3, IGFR, TGF- α , Heregulin (NDF), p-ERK, p-AKT, and p-S6 ribosomal protein or phosphorylation levels were quantified using alkaline phosphatase or peroxidase techniques and microscope-based 5 image analysis of immunohistochemically stained slides (as described in Bacus *et al.*, 1997, *Analyt. Quant. Cytol. Histol.* 19: 316-328). Quantification was by means of a CAS 200 image analyzer, as previously described (Bacus & Ruby, 1993, *Pathol Annu*, 28: 179-204). For the purpose of the analysis, tumors were classified as negative or positive for each antibody based upon the level of staining. Statistical analysis was performed using Systat to 10 quantify frequencies and calculate Pearson Chi-squared tests of significance for interactions between variables. In all cases, the p value refers to the significance of the deviation of the distribution of samples from what would be expected based upon the overall population distribution. Comparisons were performed only on samples for which all relevant data were available. As a result, the number of patients included in most comparisons was slightly less 15 than the total number of available samples.

Quantitative immunohistochemistry (IHC) was performed as previously described (Bacus *et al.*, 1997, *Analyt. Quant. Cytol. Histol.* 19: 316-328). EGFR, and erbB-2 (HER2) immunostaining was performed using pre-diluted EGFR, and erbB-2 (HER2) antibodies from Ventana Medical Systems, Inc. (VMSI, Tucson, AZ) on the VMSI automated 20 "BenchMark" staining module as described. The VMSI "I-View" detection kit was used for all three of the VMSI pre-diluted primary antibodies according to the manufacturer's instructions. Antibodies to erbB-3 (1:10), Heregulin (1:25), and TGF- α (1:20), were also used for immunostaining using the "BenchMark" with I-VIEW detection chemistry. Antibodies to Phospho-Erk (1:100) and p-AKT (1:75) were used for immunostaining using a 25 labeled streptavidin peroxidase technique as described by the manufacturer. Phospho-Erk and p-AKT slides were antigen retrieved as described by Bacus *et al.* (1997, *Analyt. Quant. Cytol. Histol.* 19: 316-328). Slides were placed onto the Autostainer (Dako Corp.) and the "LSAB2" kit (Dako) employed as the detection chemistry. After staining, all slides were counterstained manually with 4% ethyl green (Sigma). Investigators preparing and analyzing 30 tissue sections were blinded to both patient tumor type and response to therapy.

For IHC, antibodies to EGFR and erbB-2 were from Ventana Medical Scientific Instruments (VMSI) (Tucson, AZ); anti- p-AKT (Ser 437) and p-Erk1/2 were from Cell Signaling Technology Inc. (Beverly, MA); antibodies to TGFr α , erbB3, heregulin, and IGFR-1 were from NeoMarkers.

5

EXAMPLE 4

Analysis of breast cancer tissue microarrays

Tissue microarrays derived from 250 breast cancer patients who received conventional chemotherapy together with HERCEPTIN® were obtained from Clinomics Biosciences (Pittsfield, MA). The histology of the tumors varied, with infiltrating ductal carcinoma being the most common. All patients had received post-surgical radiotherapy. The tissue samples in the array were taken before treatment. HER2/neu expression had been determined by using the HercepTest system (DAKO, Carpinteria, CA) on the original biopsies for all patients. Patient response was based upon the case histories at last follow-up as decided by an independent pathologist provided by Clinomics.

Demographics of these patients are reported in Table 1. The great majority of patients had infiltrating ductal carcinomas and received anthracycline plus cyclophosphamide. Fifty-seven of the patients had metastatic diseases. All patients had received a 4 mg/kg HERCEPTIN® loading dosage and a 2 mg/kg weekly maintenance dosage.

From the original tissue arrays of 250 patients, seventy-five samples were not used in the analysis because of the lack of clinical data or because the sections did not contain useable tumor tissue. Overall, 15% of the remaining patients were disease free or had stable disease after therapy, while 85% relapsed. Of these remaining one hundred and seventy five patients, twenty-eight samples lacked HercepTest results and were therefore also excluded from further analysis. Of the samples for which HercepTest results were obtained, seventy-seven had a HercepTest score of +3, and seventy had +2 or less staining intensity (Table I).

The HercepTest staining scores were confirmed by analyzing HER2/neu expression levels using microarrays (data not shown). HER2/neu expression strongly correlated with patient response; 100% of the 0 or +1 HER2/neu patients relapsed while only 77% of the +3 patients relapsed. This response rate is similar to what has been reported previously (see

Baselga, 2002, *Annals of Oncology* 13: 8-9). Based on these results, further analysis of biomarkers concentrated on patients that expressed HER2 at the highest (+3) level. Of the samples that had the highest HercepTest scores (+3), seventy-four were taken from the primary tumor, two from lymph nodes, and one from an adrenal metastasis.

5 The seventy-seven patients who overexpressed HER2/neu (+3 HercepTest staining score) were analyzed for expression levels of EGFR, HER3, IGFR, and NDF/Heregulin, and TGF- α , as well as activated downstream signals p-ERK and p-AKT (phosphorylated forms of ERK and AKT) and the downstream signal of mTOR, p-S6 (phosphorylated S6 ribosomal protein). The analysis of receptor kinases reveled that, similar to HER2/neu, EGFR
10 expression also significantly correlated with patient response (Table 2). Among the HERCEPTIN®-treated patients that over-expressed HER-2/neu, 30% of EGFR-positive patients had stable disease or were disease free, while only 9% of EGFR-negative patients did not progress. Among the seventy-seven +3 HER2/neu patients, seventy of them expressed HER3; however, HER3 expression did not significantly correlate with patient
15 response (although the low number of HER3-negative patients limits this comparison in the data set). The growth-factor receptor HER3 is thought to play an important role in downstream erbB signaling because it has a PI3-Kinase docking site and forms active heterodimers with the other erbB receptors. The expression of other growth factor receptors may also mediate patient response, either through direct stimulation or downstream pathways
20 or through transactivation of the erbB receptors.

Expression of erbB ligands, including NDF and TGF- α , also varied among patients (see Table 3). Approximately 70% of the patients expressed high levels of NDF, while approximately 57% expressed high levels of TGF- α . A significant correlation was observed between NDF levels and patient response. A very high proportion of HER2/neu overexpressing patients who were NDF-negative relapsed (91%), whereas only 62% of NDF-positive patients who overexpressed HER2/neu relapsed. In contrast, no predictive relationship was observed between TGF- α levels alone and patient response (see Table 3). The combination of TGF- α or NDF expression and EGFR over-expression, however, did positively correlate with patient response in patients overexpressing HER2/neu ($p=0.02$ and
25 $p=0.03$ respectively) (data not shown).

The activation of heterodimers of HER2 with HER3 or EGFR results in activation of the MAPK and PI3K/AKT pathways. The MAPK pathway was measured by analyzing the level of activation or phosphorylation of ERK (pERK). Analysis and comparison of the levels of activated ERK alone, among patients that overexpressed HER2/neu and who either
5 had stable disease or who relapsed, failed to demonstrate any dramatic effect of elevated pERK levels as a factor for patient response (see Table 4). Similarly, based on this analysis, AKT activation (p-AKT) alone does not appear to be a predictive marker for response among HER2-positive patients treated with HERCEPTIN® (see Table 4). Also, analysis of S6 ribosomal protein phosphorylation, which integrates multiple signals through mTOR and p70
10 S6 kinase, did not significantly correlate with patient response for patients that overexpressed HER2/neu (see Table 4). If consideration of pERK and pAKT expression is limited to those patients that expressed EGFR and HER2/neu, however, low expression of either of these signaling molecules was a significant predictor of positive response to HERCEPTIN® (Table
5).

15 To increase the predicative power of the analysis, consideration of two or more of the biomarkers were combined in a multivariate analysis to characterize the tumor. In this analysis, the observation of the combination of HER2/neu and EGFR expression with ERK activation significantly predicated response (see Table 5). For example, patients with low EGFR expression and high ERK phosphorylation failed to respond (0% response), whereas
20 patients with high EGFR expression and low ERK phosphorylation had a high response rate (42% response). Similarly, the combination of high EGFR and HER2/neu with high NDF expression or a combination of high EGFR and HER2/neu with high TGF- α expression predicted a better response compared to patients that had low expression of EGFR and the NDF ligand (data not shown). This comparison was often dramatic. For example, while
25 39% of the patients with high EGFR, HER2/neu, and NDF expression responded to therapy, none of the patients with high HER2/neu expression but low EGFR and NDF expression responded (data not shown).

30 The combination of high Her2/neu expression, low IGFR expression, and high S6 ribosomal protein phosphorylation gave high patient response (67%, Table 5). This is in contrast to patients with high HER2/neu and IGFR expression and high S6 ribosomal protein phosphorylation, a high percentage that did not respond to therapy. The best combination of

markers for predicting whether patients that overexpressed HER2/neu would respond to HERCEPTIN® therapy were high NDF expression, low IGFR expression, and high S6 phosphorylation (Table 6). In contrast, none of the patients overexpressed HER2/neu and had low NDF expression and high IGFR expression responded to therapy, regardless of S6 status (Table 6). However, these results were obtained using a small sample population of these patients. In patients with high NDF, HER2/neu, and EGFR expression levels, phosphorylation of ERK correlated with a difference in response from 28% (high p-ERK) to 54% (low p-ERK) (Table 6). Similarly, those patients with low levels of p-AKT with any other combination of biomarkers that include the expression of HER2/neu and NDF, did better than those that over-express this protein (results not shown). Taken together, this data shows that HER2/neu together with its ligand and other erbB receptors and ligands, as well as other growth factor receptors play a role in HERCEPTIN® response. Importantly, analysis of a select combination of these proteins correlated with response rates that varied from 0 to 100%.

15

Example 5

Analysis of breast cancer samples

Samples from seven breast cancer patients were obtained from Yale University. The clinical history of these seven patients varied, with some given HERCEPTIN® in combination with chemotherapy as a first line therapy while others were given HERCEPTIN® as an adjuvant therapy. These seven samples were analyzed for receptor, ligand, and signaling protein expression or phosphorylation, and the results compared to the results with the tissue microarray analysis.

25 All seven patients over-expressed HER2/neu, as determined at the time of analysis with the other antibodies immunologically specific for non-HER2/neu polypeptides. The case histories of the patients varied. For example, patient #1 was given HERCEPTIN® plus docetaxel after relapsing with metastatic disease four years after initial presentation. This patient had stable disease for more than a year after commencing combination therapy.
30 Patient #7 was given HERCEPTIN® plus vinorelbine following the discovery of a solitary metastasis seven months after initial radiotherapy. After eight weeks of combination therapy

there was progression of disease. Of the seven patients, three showed response to HERCEPTIN® while the other four failed to respond (Table 7). One of the responders did not express IGFR but did express EGFR and showed positive downstream signaling. The other one of these responders expressed IGFR and EGFR but did not show active 5 downstream signaling in S6 or ERK. All of the non-responders expressed IGFR and had positive S6 phosphorylation. Two of the non-responders also expressed EGFR. These results are consistent with the results obtained from the microarray analysis. Patients with active IGFR receptors as demonstrated by IGFR expression plus S6 phosphorylation are unlikely to respond to HERCEPTIN®, while patients that lack IGFR or have IGFR but no 10 downstream signaling are more likely to respond.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

15

TABLE 1**Dem graphics**

	<u>number of patients</u>	<u>disease-free or stable disease</u>	<u>relapse</u>
all patients included in study	175	15%	85%
Histology			
infiltrating ductal carcinoma	109	17%	83%
lobular carcinoma	7	43%	57%
medullary carcinoma	3	33%	67%
metastatic breast carcinoma	19	5%	95%
papillary carcinoma	3	0%	100%
scirrhous carcinoma	3	100%	0%
tubular carcinoma	3	0%	100%
treatment following surgery (followed by Herceptin®)			
Doxorubicin	44	2%	98%
anthracycline plus cyclophosphamide	100	23%	77%
Paclitaxel	3	100%	0%
HER2/neu expression tumor			
0 or 1	17	0%	100%
2	53	8%	92%
3	77	30%	70%

5

Demographics of breast cancer patient samples.

Table 2.

patient group	n	% responders	% relapse	P value
EGFR positive	43	30%	70%	0.002
EGFR negative	23	9%	91%	
HER3 positive	70	29%	71%	0.43
HER3 negative	7	43%	57%	
IGFR positive	33	24%	76%	0.16
IGFR negative	35	40%	60%	

5

Receptor tyrosine kinase expression versus patient response. Analysis on tissue array samples for which clinical and Herceptest data was available and who over-expressed HER2/neu.

10

Table 3.

patient group	n	% responders	% relapse	P value
NDF positive	55	39%	62%	0.01
NDF negative	22	9%	91%	
TGF- α positive	38	34%	66%	0.56
TGF- α negative	29	28%	72%	

15 Receptor tyrosine kinase ligand expression versus patient response following therapy. Analysis on tissue array samples for which clinical and Herceptest data was available and who over-expressed HER2/neu.

Table 4.

patient group	n	% responders	% relapse	P value
p-ERK positive	36	25%	75%	0.43
p-ERK negative	39	33%	67%	
p-AKT positive	24	25%	75%	0.53
p-AKT negative	53	32%	68%	
p-S6 positive	27	33%	67%	0.74
p-S6 negative	44	30%	70%	

5

Downstream protein activation versus patient response following therapy. Analysis on tissue array samples for which clinical and Herceptest data was available and who over-expressed HER2/neu.

10

Table 5.

patient group	n	% responders	% relapse	P value
EGFR pos/p-ERK pos	21	14%	86%	0.04
EGFR pos/p-ERK neg	19	42%	58%	
EGFR neg/p-ERK pos	9	0%	100%	
EGFR neg/p-ERK neg	14	14%	86%	
EGFR pos/p-AKT pos	17	18%	82%	0.07
EGFR pos/p-AKT neg	26	38%	62%	
EGFR neg/p-AKT pos	5	20%	80%	
EGFR neg/p-AKT neg	18	6%	94%	
IGFR pos/p-S6 pos	13	8%	92%	0.01
IGFR pos/p-S6 neg	20	35%	65%	
IGFR neg/p-S6 pos	12	67%	33%	
IGFR neg/p-S6 neg	23	26%	74%	

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Analysis of receptor and downstream protein activation versus response in patients following therapy. Analysis on tissue array samples for which clinical and Herceptest data was available and who over-expressed HER2/neu.

Table 6.

patient group	n	% responders	% relapse	P value
NDF neg/p-S6 pos/IGFR neg	2	50%	50%	0.003
NDF neg/p-S6 neg/IGFR neg	9	11%	89%	
NDF neg/p-S6 neg/IGFR pos	4	0%	100%	
NDF neg/p-S6 pos/IGFR pos	4	0%	100%	
NDF pos/p-S6 pos/IGFR neg	7	100%	0%	
NDF pos/p-S6 neg/IGFR pos	16	44%	56%	
NDF pos/p-S6 neg/IGFR neg	14	36%	64%	
NDF neg/p-ERK pos/EGFR neg	3	0%	100%	0.08
NDF neg/p-ERK neg/EGFR neg	4	0%	100%	
NDF neg/p-ERK neg/EGFR pos	10	20%	80%	
NDF neg/p-ERK pos/EGFR pos	6	0%	100%	
NDF pos/p-ERK pos/EGFR neg	5	0%	100%	
NDF pos/p-ERK neg/EGFR pos	13	54%	46%	
NDF pos/p-ERK neg/EGFR neg	6	17%	83%	
NDF pos/p-ERK pos/EGFR pos	18	28%	72%	

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Analysis of ligand and receptor expression and downstream protein activation versus patient response in patients following therapy. Analysis on tissue array samples for which clinical and Herceptest data was available and who over-expressed HER2/neu.

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Table 7.

Patient	IGFR	EGFR	p-S6	p-AKT	p-ERK	Response
#1	+	+	-	-	-	yes
#2	-	+	+	+	+	yes
#3	+	+	-	+	-	yes
#4	+	-	+	+	+	no
#5	+	+	+	+	-	no
#6	+	-	+	+	-	no
#7	+	+	+	+	+	no

Receptor tyrosine kinase expression, downstream protein activation and patient response to
15 therapy in seven breast cancer patients. Analysis was of whole tissue sections.

Table 8.

AKT (NP 005154 GI:4885061) 480 AMINO ACIDS (SEQ ID NO:1)

See, e.g., Staal, S.P., Proc. Natl. Acad. Sci. U.S.A. 84 (14),
5 5034-5037 (1987).

MSDVAIVKEGWLHKRGEYIKTWRPRYFLLKNDGFIGYKERPQDVDQREAPLNNFSVAQCQL
MKTERPRPNTFIIRCLQWTTVIERTFHVTPEEREETTAIQTADGLKKQEEEEMDFRSGS
PSDNSGAEMEVEVSLAKPKHRVTMNEFEYLKLLKGKGTFGKVILVKEKATGRYYAMKILKEVI
10 VAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVMNEYANGELFFHLSRERVFSED
RARFYGAEVASALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKEGIKDGA
15 MKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVYEMMCGRLPFYQNQDHEKL
FELILMEEIRFPRTL GPEAKSLLSGLLKKDPKQRLGGGSEDAKEIMQHRRFFAGIVWQHV
YEKKLSPPFKPQVTSETD TRYFDEEFTAQMIIITPPDQDDSMECVDSERRPHFPQFSY
SASSTA (SEQ ID NO:1)

S6 (NP 001001, GI:17158044) 249 AMINO ACIDS (SEQ ID NO:2)

See, e.g., Pata et al., (1992) Gene 121 (2), 387-392.
MKLNISFPATGCQKLIEVDDERKLRTFYEKRMATEVAADALGEEWKGYVVRISGGNDKQGFP
20 MKQGVLTGHRVRLLSKGHSCYRPRRTGERKRKSVRGCIVDANLSVLNLVIVKKGEKDI
PGL TDTTVPRRLGPKRASRIRKLFNLSKEDDVRQYVVRKPLNKEGKKPRTKAPKI
QRLVTPRVLQ HKRRRIALKQRTKKNKEEAAEYAKLLAKRMKEAKEKRQEQA
25 KRRRLSSLRASTSKSESSQ K (SEQ ID NO: 2)

ERK (XP 055766, GI:20562757) 379 AMINO ACIDS (SEQ ID NO:3)

See, e.g., Butch et al., J Biol Chem., 1996., 271(8):4230-5.

30 MAAAAAQGGGGEPRRTEGVGPGVPGEVEMVKGQPFDVGPRYTQLQYIGEGAYGMVSSAYDH
VRKTRVAIKKISPFEHQTYCQRTLREIQILLRFRHENVIGIRDILRAS
TEAMRDVYIVQDL METDLYKLLKSQQLSNDHICYFLYQILRGLKYIHSANVLH
RDLKPSNLLINTCDLKICDFG LARIADPEHDHTGFLTEYVATRWYRAPE
35 IMLNSKGYTKSIDIWVG CILAEMLSNRPI FPGK HYLDQLNHILGILGSPSQED
LNCIINMKARNYLQSLPSKTKVAWAKLFPKSDSKALDLLRM LTFNPNK
RITVEEALAHPYLEQYYDPTDEPV AEEPFTFAME LDDLPKERL
KELIFQETARFQ PGVLEAP (SEQ ID NO:3)